THE PHOTOSYNTHETIC RHYTHM OF ACETABULARIA CRENULATA. I. CONTINUOUS MEASUREMENTS OF OXYGEN EXCHANGE IN ALTERNATING LIGHT-DARK REGIMES AND IN CONSTANT LIGHT OF DIFFERENT INTENSITIES [1]

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Until recently, most investigators of biological rhythms have concerned themselves with drawing inferences about the properties of the intrinsic timing mechanism from measurements of some physiological or developmental process. The lack of any more direct experimental approach has largely frustrated attempts to achieve an understanding of the "clock" at the molecular level.

This paper presents continuous records of the rate of oxygen exchange by *Acetabularia* cells over periods of several days to one week. A recently developed polarographic system employing a graphite cathode was used to monitor oxygen metabolism. During the experiments pretrained cells were exposed to alternating light-dark regimes and to continuous light of different intensities. A second paper seeks to reveal the point at which control is exercised by analyzing the time-dependence between the rate of photoassimilation of CO_2 and the activity of selected enzymes in the reductive pentose phosphate pathway (Hellebust, Terborgh, and McLeod, 1967).

The unicellular alga *Acetabularia* possesses a number of properties which make it a particularly suitable subject for an investigation of the means of control of a rhythmic process. It exhibits a prominent circadian rhythm of photosynthesis expressed both in CO_2 uptake (Richter, 1963) and in O_2 evolution (Schweiger *et al.*, 1964) which persists under constant conditions, even in the absence of its nucleus (Sweeney and Haxo, 1961). Of further relevance are the facts that it grows by cell enlargement without any apparent differentiation for a period much longer than that required for expression of the rhythm, and that measurements on single cells are technically simple (Schweiger *et al.*, 1964).

Materials and Methods

Experimental material consisted of groups of intact *Acetabularia crenulata* Lamarous cells in the phase of stalk elongation (Terborgh and Thimann, 1965).

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Cultures were maintained in a rapid state of growth in cotton-stoppered 500-ml. Erlenmeyer flasks containing Erdschreiber solution. Unless otherwise stated, cultures were given an alternating, 8-hour-16 hour, light-dark regime for at least two weeks prior to experimentation. Illumination of 300 ft.-c. was provided by "Daylight" fluorescent tubes during the light periods. The temperature was held at approximately 28° C. throughout the training cycles and during experimentation.

The rate of oxygen exchange by samples of 20 to 50 cells of 0.5 to 1.0 cm. in length was measured by means of the open polarographic system described by McLeod *et al.* (1965). The device measures the relative oxygen tension in a cup-shaped sample chamber 1 cm. in diameter. The chamber is lined below with a graphite paste that serves as the cathode in the oxygen reaction at a polarizing potential at 0.64 V.

The cathode chamber was covered above with a transparent dialysis membrane that held the algal sample in place, the whole device being immersed in a bath of Erdschreiber medium that doubled as an electrolyte. The anode was a Ag-AgCl bar located in the bath 3 cm. from the cathode chamber. Current flow between the electrodes was proportional to the oxygen tension in the cathode chamber. Signals fell in the range of 0 to 2 μamps and were amplified by a General Radio Model 1230-A electrometer and recorded continuously on a Varian G-14 or a Sargent SR recorder operated at very low chart speeds.

Readings obtained from a sample of cells in the dark reflect the concentration of oxygen in the cathode chamber during respiration. Since the solution around the sample is allowed to exchange freely with the bath, any constant rate of metabolic activity will in time establish a steady-state balance between the rate of oxygen exchange in the tissue and the rate of oxygen diffusion through the dialysis membrane. After major (on-off) changes in the illumination of the cells, complete establishment of a new steady-state of gas exchange requires $\frac{1}{2}$ to 1 hour, though about three-quarters of the change takes place in the first 5 to 10 minutes. Illumination of an algal sample thus results in a sharply rising trace.

The electrode assembly was housed in a light-tight wooden box which had a slide projector mounted outside on the end wall. The collimated beam was directed into the cathode chamber containing the algal sample by means of first-surface mirrors. Its intensity was controlled witht a variable transformer. Readings from a GE foot-candle meter (Model No. 213) at a level comparable to that of the sample chamber under appropriate layers of Erdschreiber medium and dialysis membrane were taken as estimates of the intensity incident on the algal samples at different Variac settings. Further details, including a diagram of the electrode assembly and optical system, are given by Terborgh (1966).

Results

Oxygen metabolism of Acetabularia crenulata in alternating periods of light and darkness

Continuous records of oxygen exchange by groups of *Acetabularia* cells in an alternating light-dark (LD) regime reveal a complicated time course in light that consists of two peaks of photosynthetic output (Fig. 1).

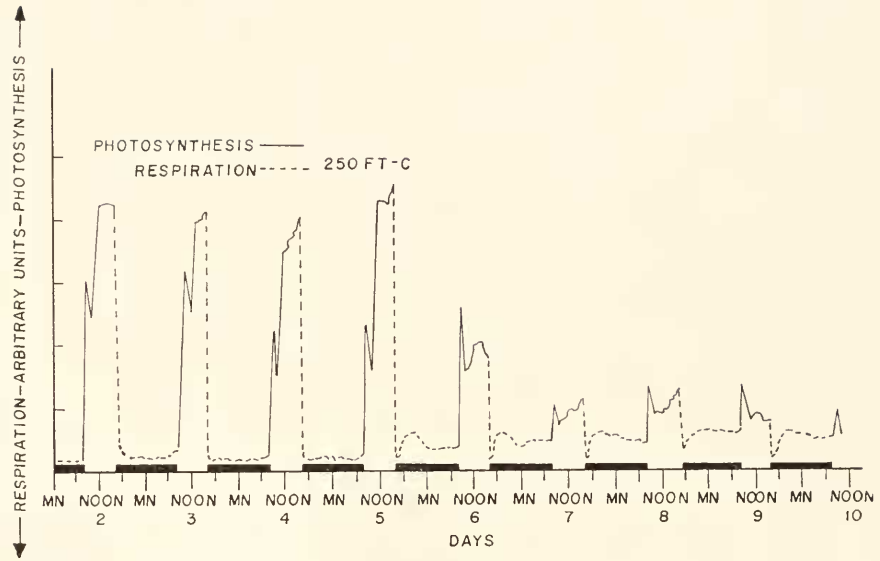

FIGURE 1. Oxygen exchange by *Acetabularia* cells in a 24-hour regime composed of 8 hours of light at 250 ft.-c. and 16 hours of darkness. Ambient temperature was 28° C. Heavy lines on abscissa denote dark periods. Decreased performance after day 5 was attributed to a gradually increasing toxicity of the electrode environment.

The first (stage 1) appears immediately on illumination and in approximately 30 minutes is followed by a sharp depression. A second rise (stage 2) begins after about 1 hour and develops a higher maximum after 4 to 8 hours.

This second upswing in the traces continues for the remainder of the light period. The prolonged gradual rise that is observed in this portion of the records is slow in relation to the response time of the measuring system, and so represents closely the actual time course of the photosynthetic output of the sample. Maximum rates were generally attained in the latter part of the daily light periods, often in the final hours.

On extinguishing the light, the concentration of oxygen in the sample chamber dropped rapidly for about 30 minutes and then decreased gradually until a respiratory steady-state had been established. Nearly steady traces were the rule during 16-hour dark periods in phase with the entraining regime.

The electrode environment did not appreciably affect the performance of algal samples for the first 4 or 5 days of recording. Subsequent progressive decreases in both photosynthetic and respiratory activity indicated the onset of deleterious conditions, possibly resulting from a gradual accrual of toxic levels of silver ion in the medium.

Partial specificity of this inhibition was apparent in that all parts of the cycle were not equally affected. The loss of activity in stage 2 subsequent to day 5 in Figure 1 was much more pronounced than the reduction of stage 1, the maximum photosynthetic rate then being found in the latter. This result thus suggests that the two stages are inhibited by different processes.

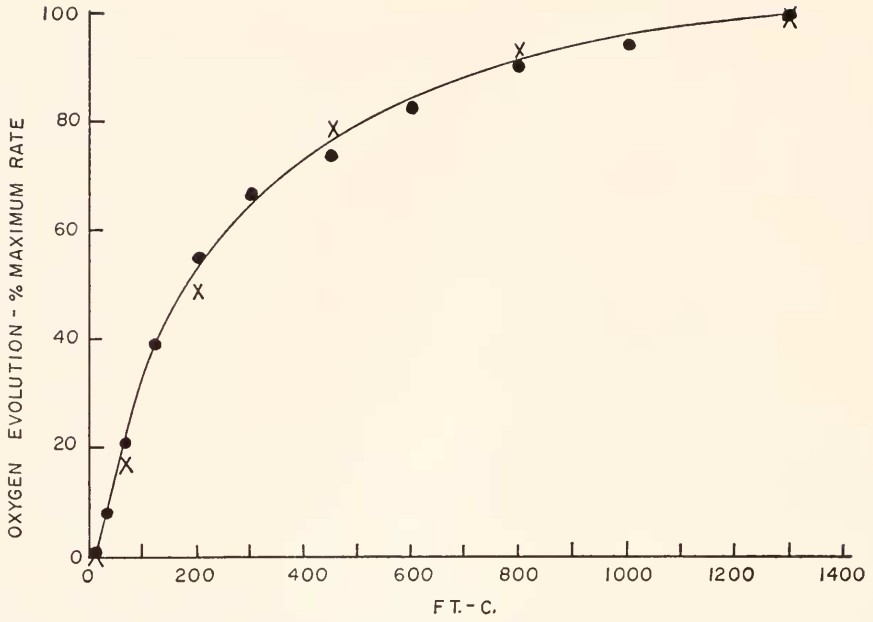

FIGURE 2. Photosynthesis as a function of the intensity of light of *Acetabularia* cells grown in a regime of 8 hours of light at 300 ft.-c. and 16 hours of darkness. Experimentation was carried out in the latter half of a light period. Performance at each intensity was taken as the increase in rate of oxygen production recorded over 30 seconds of illumination after the cells had established a steady-state of oxygen exchange in the dark. O = increasing intensity series, X = decreasing intensity series.

The effects of the electrode environment may result in a much-altered time course of respiration in the dark periods. Under these conditions, the respiratory cycle assumes a form similar to that of photosynthesis, consisting of a transitory early maximum followed by a slowly developing second stage of more rapid activity. As was the case for oxygen evolution, the initial burst of respiratory oxygen consumption appeared to be less sensitive to inhibition than the later stage. The first period of inhibited respiration came after the fifth day of the experiment, during which photosynthesis was normal, indicating that substrate limitation was probably not the cause.

The light curve of Acetabularia crenulata

Endogenous regulation of a photosynthetic rhythm could be exercised at the level of either the light or dark reactions, or alternatively through alterations in the tightness with which the light and dark reactions are coupled. One may distinguish between at least the first two of these possibilities by determining the effect of different light intensities on the expression of the free-running rhythm. The interpretation of such experiments is based on the characteristics of a photosynthetic light curve such as the one presented in Figure 2. The cells used in

producing the curve were grown in an 8L–16D regime and subjected to experimentation in the latter half of a light period. The physiological state of the cells was therefore that of late stage 2 when the diurnal photosynthetic cycle is at its maximum. Before each exposure to light the algae were allowed to reach a steady-state of oxygen exchange in the dark. The rate of photosynthesis at each intensity was then taken as the increase in the rate of oxygen production that took place during 30 seconds of illumination. Since we observed stage 1 responses only following dark periods of at least several hours' duration, the light curve in Figure 2 is attributable to stage 2 photosynthesis.

The light curve obtained in this manner has an I_k somewhat below 300 ft.-c. whereas saturation required intensities in excess of 1000 ft.-c. I_k is the intensity at which the extrapolated initial slope of a light curve intersects a horizontal line through the maximum rate of photosynthesis. Curves of this form are produced even by single *Acetabularia* cells (unpublished results), eliminating the possibility that mutual shading was responsible for the unusually slow approach to saturation. Consequently, the rate of photosynthesis of *Acetabularia* over a wide range of intermediate light intensities is not limited solely by a light reaction or a dark reaction, *sensu strictu*.

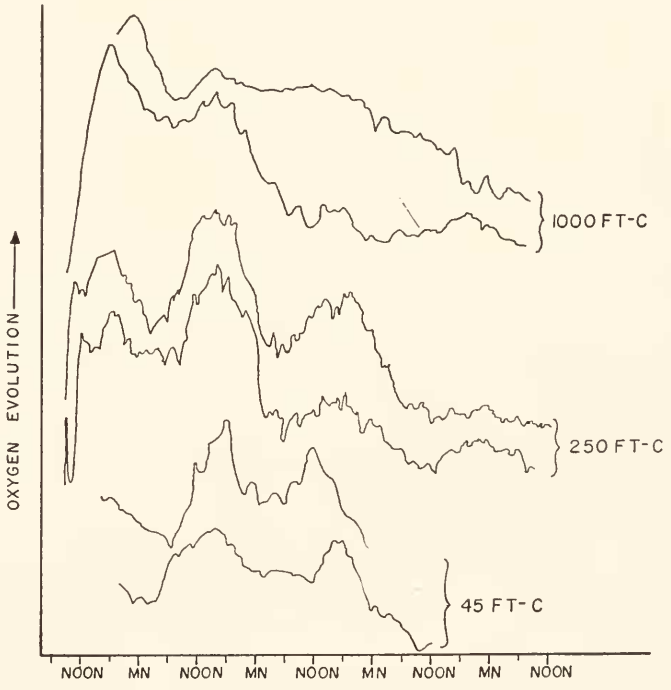

FIGURE 3. The free-running rhythm of oxygen production in continuous light at three intensities. Scale of ordinate is non-uniform. Prior to experiments the cells received 8 hours of illumination at 300 ft-c. daily from 9 to 17 o'clock.

Stages 1 and 2 of the diurnal cycle at different light intensities

Having shown that stages 1 and 2 of the diurnal photosynthetic cycle appear to be limited by different processes, an attempt was made to further characterize them by testing the response to step-up increases in light intensity at different times in the cycle. Estimates of the maximum photosynthetic capacity were obtained at intervals throughout the day by raising the light intensity from 250 to 1000 ft.-c. for periods of 90 seconds. The time course of photosynthesis revealed by the high-intensity spot checks essentially paralleled that observed at 250 ft.-c. However, the relative rate increases stimulated by the 1000 ft.-c. irradiations were appreciably greater during the maxima of stages 1 and 2 than during the depression between them. At this time the capacity of the cells appears to have been reduced to a greater extent than their activity.

Expression of the free-running rhythm in continuous light of different intensities

Regular oscillations in the photosynthetic output of *Acetabularia* cells exposed to continuous light persist for many days. This circadian rhythm is conspicuously expressed over a range of light intensity that encompasses the three major sections of the light curve (Fig. 3). Preceding the beginning of a continuous light regime the cells received 8 hours of illumination at 300 ft.-c., followed by a dark period. Experiments began at the start of the subsequent light period at near saturation (1000 ft.-c.), at an intermediate level (250 ft.-c.), and at an intensity on the linear portion of the light curve (45 ft.-c.). At all three levels, but especially at the highest, the daily photosynthesis decreased during the course of the experiments. The electrode microenvironment may have contributed to this behavior, but it could also represent a physiological adaptation to continuous light in cells preconditioned to 8-hour photoperiods.

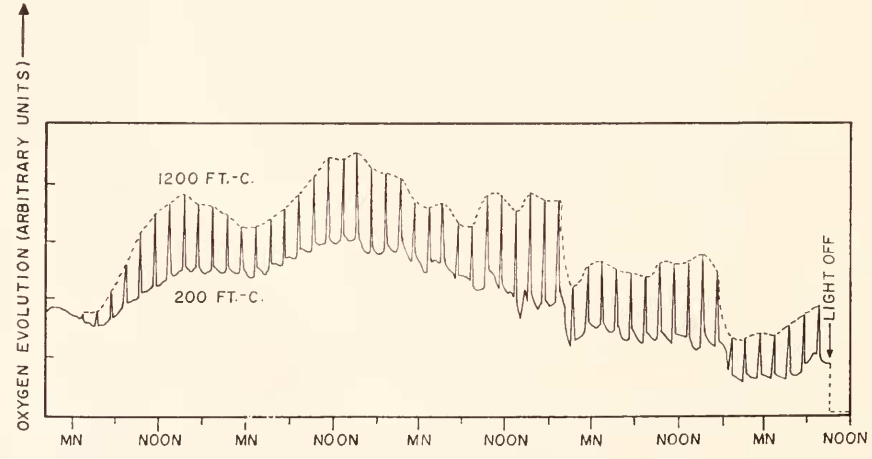

FIGURE 4. The free-running rhythm of oxygen production in continuous illumination of 250 ft.-c. on which 15-minute exposures to 1000 ft.-c. were superimposed every 2 hours.

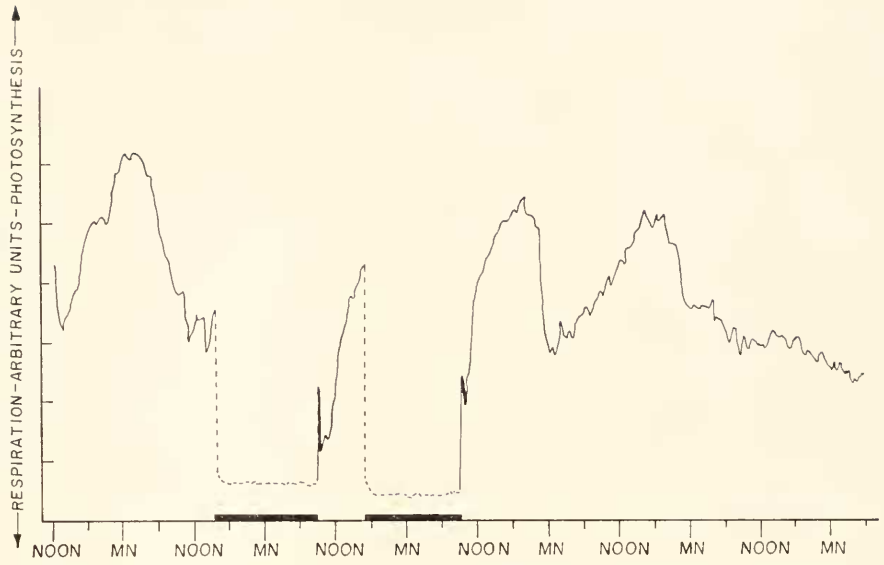

FIGURE 5. The effect of a single 8-hour light period on the phase of the rhythm of oxygen production. Prior to the start of the experiment the cells had been in continuous light for 1 week, during which the time of maximum output had shifted to 1 to 2 o'clock in accordance with a free-running period somewhat in excess of 25 hours.

The amplitude of the circadian oscillation diminishes rapidly under high-intensity illumination, but the vestige of the rhythm that remains after 3 or 4 days indicates that its period is unaffected. The free-running rhythm is exhibited with maximum amplitude at intermediate light intensities. In none of these experiments was there any indication that either the period or the phase of the free-running rhythm was affected by light intensity.

In order to establish that the photosynthetic apparatus was not adapting to whatever light intensity was being used to demonstrate the free-running rhythm, experiments were programmed to give indications both to the capacity and activity of photosynthesis throughout the cycle (Fig. 4). This was accomplished by giving 15-minute exposures to high intensity (1000 ft.-c.) illumination every 2 hours on a continuous background of moderate intensity (250 ft.-c.). The maximum rates recorded at the lower intensity did not exceed the minima in the high intensity cycle. This result clearly shows that the maximum photosynthetic capacity of *Acetabularia* remains well above the range of the oscillations in activity observed at low or intermediate intensities. It is evident that capacity and activity vary simultaneously, and that the former undergoes changes of greater amplitude.

Resetting the rhythm of photosynthesis

Figure 5 illustrate an experiment performed with cells that had previously been held in continuous illumination of 300 ft.-c. for one week after having been entrained

with the usual 9 to 17 o'clock light periods. The first day's record shows that the free-running rhythm had persisted with undiminished amplitude through a week devoid of recurrent stimuli. The maximum in the cycle now came at 1 to 2 o'clock instead of at 17 to 19 o'clock, indicating the free-running period is not exactly 24 hours in constant conditions.

With the commencement of a second cycle of photosynthetic output, the cells were given 18 hours of darkness followed by a 9 to 17 o'clock training period. The free-running rhythm in 250 ft.-c. subsequently attained maxima around 18 o'clock in the manner of cells that had received many 9 to 17 o'clock entraining periods (*e.g.*, Fig. 3). A single light-dark cycle thus suffices to reset rhythmicity.

In the experiment of Figure 5 the response to illumination during the 8-hour training period was exceptionally poor even though it was given during a portion of the cycle when the photosynthetic performance should have been falling. Oxygen production in the entraining period did not reach the minimum level of the preceding or subsequent free-running cycles for 2½ hours. Depression of photosynthesis in out-of-phase light periods occurred to varying degrees in other experiments of this kind, though usually less than in the present instance.

Discussion

In constant illumination at 28° *Acetabularia* cells exhibit a free-running rhythm of oxygen production. Continuous records of the photosynthetic output over 8-hour light periods that were in phase with the entraining regime showed a time course consisting of two distinct stages. Upon illumination a rapid rate of oxygen production commenced immediately as indicated by the sharp initial rise of the traces. This is conspicuous only following periods of darkness of several hours, and thus may be dependent on the gradual accumulation of a rate-limiting substrate. The initial burst of oxygen production is followed by a depression and a subsequent slow rise to a second maximum (stage 2).

The gradual rise and fall of photosynthetic output in constant light follows kinetics similar to those of stage 2 of the diurnal time course and suggests that the two oscillations share the same mechanism. Since stage 1 is a direct consequence of long-continued darkness, it should not be confused with the manifestations of rhythmicity in stage 2 of LD schedules and continuous light.

That different reactions limit the rate of oxygen production during stages 1 and 2 is attested by their differential sensitivity to the deleterious effects of long-term exposure to the electrode environment. Investigation of the possible periodic expression of stage 1 would be of interest in reference to the two-component model for a temperature-compensated rhythm proposed by Hastings and Sweeney (1957).

The photosynthetic rhythm of *Acetabularia* differs from that of some other unicellular algae in being expressed, for several cycles at least, in continuous light at any intensity within the range of 45 to 1000 ft.-c. The rhythms of luminescence, photosynthesis and cell division in the marine dinoflagellate *Gonyaulax polyedra* are all promptly suppressed by light intensities of 800 to 1000 ft.-c. (Hastings *et al.*, 1961; Hastings and Sweeney, 1964). This result holds for photosynthesis assayed as carbon dioxide fixation (Hastings *et al.*, 1961) or oxygen evolution (Sweeney, 1960). In continuous dim light (100–200 ft.-c.) rhythmicity of lumines-

cence and cell division persist in *Gonyaulax*. The rhythm, which involves the capacity for photosynthesis, is not observable until the capacity is tested by exposing the cells briefly to bright light (1000 to 1500 ft.-c.). Thus Hastings *et al.* (1961) concluded that only the maximum photosynthetic capacity of *Gonyaulax* was affected. Determination of light curves for oxygen evolution by single *Gonyaulax* cells at different times of day led to the same conclusion (Sweeney, 1961). Such a clear interpretation cannot be afforded the work of Palmer *et al.* (1964) who found that circadian oscillations in carbon dioxide fixation by the diatom *Phaeodactylum tricornutum* persisted for at least 3 cycles under continuous illumination at 80 ft.-c. but quickly damped at 20 and 600 ft.-c. Sweeney has shown that the activity of ribulose-diphosphate carboxylase in *Gonyaulax* extracts varies with the same period and amplitude as the rhythm of photosynthesis (1964, 1965).

When *Acetabularia* is brought into continuous light from an LD regime (8 hours at 300 ft-c. and 16 hours darkness in these experiments) the daily photosynthesis is at first high but tends to drop within two or three days to considerably lower levels. This is generally accompanied by a reduction in the amplitude of the rhythm and is particularly rapid and pronounced at high light intensity. To some extent this effect may be attributable to deleterious effects of the electrode environment, but in control experiments there was no reduction of photosynthetic output in LD until after the fifth day of experimentation. Thus the observed decreases in photosynthesis under continuous illumination may be largely adaptive.

This notion is supported by the results of earlier experiments in which cells given 8-hour photoperiods grew more slowly at both high and low intensities and contained up to 3 times as much chlorophyll as those given 16-hour photoperiods or continuous light (Terborgh and Thimann, 1964). It is apparent from these results that when *Acetabularia* is transferred from an 8-hour light regime to continuous light there follows a considerable reduction in chlorophyll content over the next few days. Thus the gradual loss of productivity that characterizes our records of the free-running rhythm, to a degree at least, must be attribuable to concomitant adaptive reductions in chlorophyll content. On the other hand, the possibility that diurnal fluctuations in chlorophyll content contribute to the expression of the photosynthetic rhythm has been thoroughly examined with the result that no such variations are discernible (Hellebust *et al.*, 1967). Adaptive changes in chlorophyll concentration in response to altered conditions require periods in excess of 24 hours in *Acetabularia* and so cannot account for any part of the photosynthetic rhythm. Reported diurnal cycles in the chlorophyll content of the leaves of a number of higher plants entail concentration changes of only 20% or less and therefore could not in themselves produce a photosynthetic rhythm of large amplitude (Wendel, 1957; Bünning, 1959). Parallel oscillations in photosynthesis and chlorophyll content of some natural phytoplankton communities (Yentsch and Ryther, 1957) are subject to a variety of interpretations (Steemann Nielsen and Jorgensen, 1962). Even in *Chlorella*, the adaptive adjustments in chlorophyll content that follow step-up or step-down changes in light intensity are much less rapid than those that would be necessary to mediate a circadian rhythm in photosynthesis (Steemann Nielsen *et al.*, 1962).

The fact that the photosynthetic rhythm of *Acetabularia* is expressed at both high and low light intensities does not permit a simple interpretation. One possi-

bility is a time-keeping mechanism that separately and simultaneously controls rate-limiting steps in both the light and dark processes. Detracting from the credibility of this view, however, are the facts that *Acetabularia* undergoes no cyclic variation in chlorophyll content and that extensive experimentation has failed to reveal a potentially rate-limiting step in the Calvin cycle, though the activities of some 9 enzymes have been examined (Hellebust *et al.*, 1967). A simpler alternative would be a control system that operates at the biochemical level at which the light and dark processes are coupled. The notion of metabolic plasticity at this point can also be implied from the slowness with which the light curve reaches saturation. Linearity holds only to 150 or 200 ft.-c. in contrast with *Gonyaulax* in which it extends to 500 to 800 ft.-c. depending on the time of day (Sweeney, 1960). Clearly, more substantive evidence from this difficult area in metabolism is required before the rate-limiting reaction(s) can be pinpointed.

Our finding that a single 8-hour light period suffices to reset the phase of the photosynthetic rhythm in *Acetabularia* is in good accord with results obtained with other photosynthetic organisms. For instance, a 12-hour period of illumination resets the time of maximum phototactic responsiveness of *Euglena* (Bruce and Pittendrigh, 1956). Even more sensitive is *Gonyaulax* which can be rephased by a single exposure to altered light intensity. The number of hours of phase shift produced depends on both the intensity and duration of such treatments (Hastings and Sweeney, 1958).

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Summary

1. Oxygen exchange by *Acetabularia crenulata* in various light regimes was monitored continuously for as long as 10 days by means of a rate-measuring graphite oxygen electrode.

2. The time course for photosynthesis in 8-hour light periods is bimodal, and consists of an initial burst of oxygen production followed by a depression and a subsequent slow rise to a (usually) higher maximum in the latter part of the period. The two maxima show differential sensitivity to the deleterious effects of long-term exposure to the environment of the oxygen electrode.

3. The light curve for photosynthesis departs from linearity at the comparatively low intensity of 200 ft.-c. but does not reach saturation below 1300 ft.-c., indicating an unusually loose coupling of the light and dark reactions.

4. Both maxima of the diurnal time course of oxygen evolution as well as the free-running rhythm were expressed at a moderate (250 ft.-c.) and at a high (1000 ft.-c.) light intensity. A free-running rhythm was also found at 45 ft.-c. The possibility that only the maximum capacity of photosynthesis fluctuates in the expression of the rhythm was ruled out in an experiment that monitored both capacity and activity alternately in the same cycles.

5. The natural period of the rhythm at 28° C. is approximately 25 hours. The phase can be reset by a single 8-hour photoperiod.

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